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PATENT & TRADEMARK OFFICE

Attorney's Docket No.: 23048-004006/402E

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Gyula Hadlaczky et al.
Serial No. : 09/724,726
Filed : November 28, 2000
Conf. No. : 7776
Title : *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*

Art Unit : 1638
Examiner : Brent T. Page
Cust No. : 20985

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Steven F. Fabijanski declare as follows:

1) I am familiar with the subject matter of the above-captioned application, which was filed on November 28, 2000, as well as the parent applications, including the earliest application U.S. Application Serial No. 08/629,822.

2) I have reviewed the Office Action, mailed July 31, 2007, in connection with the above-captioned application.

3) I received a Bachelor's degree in Biology from the University of Miami (Florida) in 1977. I received a Ph.D. degree in Cellular and Molecular Biology from the University of Southern California in 1981. I have held post-doctoral positions at the University of Ottawa in Ottawa, Ontario, Canada and the University of Southern California in Los Angeles, Califonia from 1982 to 1985. From 1986 to 1991, I held the position of Research Director at Paladin Hybrids, Inc.

4) I have more than 20 years of experience in the area of plant molecular biology, plant gene expression, plant tissue and cell culture and development of techniques to produce genetically modified plants and plant artificial chromosomes. I have authored or co-authored over 20 publications and I am an inventor of over 16 US and foreign patents.

5) I currently am President & CEO of Agrisoma Biosciences, Inc., located in 8081 Lougheed Highway, Burnaby, B.C., Canada V5A 1W9. I have held this position since June 2007. Before that, I was Director of Research and Development at Agrisoma Biosciences Inc., a position I held since 2001. I also was President of the FAAR Biotechnology Group,

Inc., located at Suite 323, 5929L Jeanne D'arc Boulevard, Orleans, Ontario, Canada K1C 7K2 from the period 1992 - 2007.

6) Chromos Molecular Systems, Inc., located at 8081 Lougheed Highway, Burnaby, B.C., Canada V5A 1W9, an assignee of record, is an owner of Agrisoma Biosciences, Inc., to whom the subject matter of this application has been licensed. The licensed technology, as it relates to plant SATACs, is a cornerstone of the technologies commonly practiced by Agrisoma Biosciences, Inc. Indeed, production and use of plant SATACs is the technology upon which the commercial activities of Agrisoma are focused.

7) The Declaration pursuant to 37 C.F.R. §1.132 submitted under my signature to the U.S. Patent and Trademark Office on April 30, 2007 (referred to herein as Declaration 5) is incorporated by reference herein.

8) I have read and reviewed the comments made by the Examiner in the instant Office Action as they relate to Declaration 5. The comments appear to question the cytological techniques used to identify SATACs, and intermediates and precursor of SATACs; the images provided evidencing generation of SATACs in plants, and intermediates and precursors thereof, as using methods as described in the instant application; and the stability of SATACs. My comments below address the points raised by the Examiner.

9) To practice the method in plants, no additional experimentation or additional guidance was required to successfully generate plant SATACs beyond what was described in the application and known to one of skill in the art at the time the application was filed. The method as described in the application consistently and reproducibly results in the generation of plant SATACs in high frequency. The method has been performed in plants numerous times. Also, numerous research groups including myself, persons under my direction and other research groups that have collaborated with Agrisoma Biosciences, Inc., including the Eastern Cereal and Oilseeds Research Centre of Agriculture and Agri-Food Canada, one of the federal crop research centers of the Ministry of Canada responsible for Agriculture; the Hungarian Biological Research Center in Hungary; Chromos Molecular Systems, Inc., Burnaby, B.C., Canada; and Agrisoma's research group at the Plant Biotechnology Institute in Saskatoon, Saskatchewan, Canada, have practiced the technology exactly as described in the application to generate plant satellite artificial chromosomes (SATACs) and have successfully generated plant SATACs.

Further, the methods, when performed in the diverse plant species of *Brassica* or *Nicotiana*, as described in Declaration 5, consistently result in a frequency between 20% - 40% of the resistant events recovered (and subsequent full regenerated plants) containing SATACs or intermediates to SATACs, such as sausage chromosomes. The frequency at which SATACs or intermediates in the formation of SATACs were obtained is similar even using varied methods of DNA introduction into cells, and is not influenced by which research group was conducting the work.

Further, in each case of SATAC production in *Brassica* or *Nicotiana* as described in Declaration 5, the exact same components were used to produce a SATAC: introduction of a DNA sequence comprising a portion of the highly conserved 26S rDNA coding region and a common selectable marker (35S:PAT). In some of the work conducted, the exact same DNA mixtures from the same test tube were used for *Nicotiana* and *Brassica* SATAC formation. Hence, the results clearly indicate a commonality and universality of the method. The method of generating SATACs can be utilized to generate transgenic plants, and the efficiency and reproducibility of the method exceed all of the typical methods of transgenic plant production I have personally practiced, or supervised the practice of, in my 20 years of experience in producing transgenic plants.

10) The cytological visualization of these SATACs was conducted using standard techniques. No special skills are required, only the ability to follow well-documented protocols in the literature known at or prior to the time of the earliest priority date (see *e.g.*, Leitch *et al.* (1991) *Genome*, 34:329-333; Fukui *et al.* (1994) *Theor. Appl. Genet.*, 87:893-899; Jiang *et al.* (1995) *Proc. Natl. Acad. Sci.* USA, 92: 4487-4491; Murata and Motoyoshi (1995) *Chromosoma*, 104:39-43; Matsuyama *et al.* (1996) *Genome*, 39:941-945; Zhong *et al.* (1996) *Chr. Res.*, 4:24-28; and Schubert I and Wobus U (1985) *Chromosoma*, 92: 143-148).

11) Declaration 5 provided examples of SATACs, and precursors and intermediates of SATACs, obtained in *Nicotiana* and *Brassica*, using the method as described in the application. The images shown were merely representations of what is commonly seen in the laboratory, and represent only a very small subset of events analyzed and identified. For example, Figure 1 described the generation and analysis of what those who routinely analyze thousands of these chromosome spreads have determined to be a dicentric intermediate of a SATAC. Figure 2 depicted the generation and analysis of a sausage chromosome intermediate. Figure 3 depicted the generation and analysis of a SATAC in *Nicotiana*.

Exemplary images of a SATAC and precursors and intermediates of SATACs generated in *Brassica* also were described in Figure 4.

Thus, Declaration 5 described the generation of 20 independent SATACs or precursors and intermediates in the formation of SATACs (i.e. dicentric chromosomes or sausage chromosomes), generated in *Nicotiana*. The actual number of *Nicotiana* SATACs, or intermediates of SATACs, including sausage chromosomes, produced to date far exceed that number; the laboratory routinely isolates between 10 and 20 events demonstrating targeting to, and amplification of, heterochromatin per 100 events analyzed. Typically, 100 – 200 events are analyzed. As described in the application, simple southern blotting techniques can be used to identify events where amplification takes place, and in many instances simple chromosome spreads stained with DAPI can identify SATAC precursors such as sausage chromosomes. In addition, Declaration 5 described the generation of more than 10 SATACs, and precursors and intermediates in the formation of SATACs, generated and identified in *Brassica*. In practicing the method in *Brassica*, we routinely recover between 30 and 40 events demonstrating targeting to, and amplification of, heterochromatin per 100 events analyzed. Simple southern blotting techniques are again used to identify events where amplification takes place and subjected to further analysis such as common cytological techniques such as FISH. The results demonstrate that the SATACs and methods for producing as described in the above-captioned application and priority applications are, as described in the application, readily practiced with plants cells.

12) No knowledge of the centromere sequence is required to practice the method of the above-captioned application, and to generate SATACs, including plant SATACs. All that is required to practice the method is to insert a nucleic acid into the pericentric heterochromatin, whether by random introduction or by targeting the nucleic acid to the pericentric heterochromatin. Duplication of the centomere as part of the amplification of the heterochromatin provides all of the necessary elements for centromere function in a SATAC. Thus, the plant SATACs provided in Declaration 5 were generated without a singular iota of knowledge of the molecular components or configuration of the centromere itself.

13) Declaration 5 also presented evidence of the maintenance, stability, and function of the *de novo* formed centromere found within plant SATACs. For example, the Declaration 5 showed that in generating SATACs in *Nicotiana*, the selection and analysis of the tobacco protoplasts was performed 14 to 21 days after transfection, meaning thousands of mitotic divisions occurred and the SATAC was still maintained.

The present Declaration provides additional data evidencing the stability of SATACs generated in plants. This Declaration describes the meotic stability of a plant SATAC in *Brassica napus* by analysis of root tips from seed produced by a regenerated plant carrying a SATAC. The SATACs generated in *Brassica napus* exhibit a typical segregation and displayed homozygous, hemizygous and null genotypes at a predicted Mendelian frequency. The function of the centromere is clearly ascertained by the presence of a SATAC in growing and dividing plant cells. There is no better evidence of the functionality and stability of a centromere associated with a plant SATAC than its persistance from generation to generation in seed plants and, demonstrating true Mendelian inheritance patterns. There is no other definitive proof of centromere function than appropriate adherance to genetic principles.

A description of the above-referenced methods and the resulting production of plant SATACs, including evidence of their maintenance is described in the following sections.

I. MATERIALS AND METHODS

Generation of Plant SATACs in *Brassica napus*

Using methods as set forth in the above-referenced application, heterologous DNA was introduced into *Brassica napus* to produce plant SATACs, following selection and amplification of pericentric DNA. This is described in Declaration 5, incorporated by reference herein. Briefly, the heterologous DNA included a construct containing a CaMv 35S promoter fused to a phosphinothricin acetyl transferase gene (*bar)* as a selectable marker (White *et al.* (1989) *Nucleic Acids Res.*, 18:1062), with an *att B* recombination site between the 35S promoter and *bar* selection gene, and was contained in a pBluescript backbone (Stratagene, La Jolla, CA), referred to as pABI 012. The DNA encoding the *bar* selectable marker was introduced along with 26S targeting DNA (Genbank Accession no. X52320, which was deposited in the early 1990s; see also Pruit and Meyerowitz (1991) J Mol Biol., 187:169-83; Genbank Accession no. X15550; Gruender et al. (1991) J Mol Biol. 221:1209-1222). The targeting DNA was cloned into vector pBluescript (Stratagene, La Jolla, CA).

Typically 1-10 X 10^6 mesophyl protoplasts were isolated and used for DNA uptake. *Brassica napus* protoplasts were isolated from mesophyll material derived from *in vitro* cultured shoots essentially as described by Vamling K. and Glimelius K. (see e.g., Legumes and Oilseed Crops I in Biotechnology in Agriculture and Forestry 10, Springer Verlag (1990)). Plasmid DNAs from the vector and targeting DNA were sterilized with chloroform and 70% ethanol before use for transfection. A protoplast suspension was mixed with vector and targeting DNA at a ratio of 1:10 followed immediately by slowly adding PEG solution.

Typically, 30 μg of DNA mixture (vector and targeting DNA at a 1:10 ratio) were used per 1 X 10^6 protoplasts.

Selection was on L-PPT and the plants were regenerated used standard protocols known in the art, *e.g.* those described in: Vamling K. and Glimelius K. (see *e.g.*, Legumes and Oilseed Crops I in Biotechnology in Agriculture and Forestry 10, Springer Verlag (1990); Glimelius K. *et al.* (1986) *Plant Sci.* 45, 133 – 144; Barsby *et al.* (1986) *Plant Cell Reports 5*, 101). Typically, up to 50% of L-PPT resistant calli regenerated to shoots and whole plants. *In situ* hybridization analysis was performed on regenerated plants. Specifically, the presence of a SATAC, and intermediate and precursors of SATACs, in second generation plants was ascertained by fluorescence *in situ* hybridization on root tips obtained from T1 seed that was obtained by self-pollination of T0. The seedlings were grown in the presence of L-PPT. Resistance to L-PPT is conferred by the presence of the 35S PAT gene used for the production of the SATAC. Seeds were germinated on filter paper, and emerging root tips were sampled in a non-destructive fashion, blocked and subjected to two-color FISH analysis using a 35S PAT probe (yellow-green signal) and an 18S rDNA probe (red signal). The remainder of the seedling was allowed to grow and was exposed to L-PPT.

II. Results

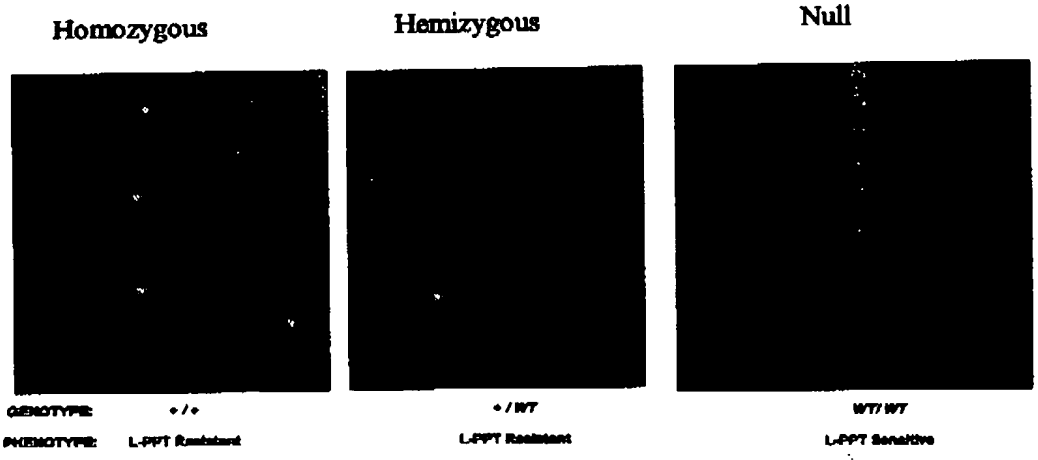
Analyses of SATACs, and precursors and intermediates of SATACs, generated in *Brassica napus* were performed on root tips from seed produced by a regenerated plant carrying a SATAC. Analysis of second generation plants was performed by fluorescence *in situ* hybridization of root tips obtained from T1 seed that was obtained by self-pollination of T0 plants. Chromosome spreads were stained with DAPI and co-stained for the presence of the selectable marker using a 35S PAT probe (yellow-green signal) and pericentric heterochromatic DNA using an 18S rDNA probe (red signal). As described in Declaration 5, multiple structures of chromosomes having identifying characteristics as described in the above-referenced application for the generation of SATACs, were observed in *Brassica*, following selection and regeneration to whole plants.

In analyzing the T1 seed, three genotypes were observed. The first being seed that were homozygous for the SATAC signal (yellow green), the second being seed that were hemizygous for the SATAC signal and the third being seed that were null for the SATAC signal. This is depicted in Figure 1 below, where arrows indicate the presence of a SATAC, or an intermediate precursor of a SATAC, in root tip spreads in the homozygous, hemizygous and null genotypes obtained from a T0 plant carrying a SATAC genotype. It

should be noted that the presence of the rDNA regions (red signal) again are found typically in the pericentric regions of 12 of the 44 chromosomes in *Brassica*. The phenotype of these genotypes was determined by growth of the seedlings in the presence of L-PPT, which was used as the selective agent. Resistance to L-PPT was conferred by the presence of the 35S PAT gene used for production of the SATAC. Resistance to L-PPT correlated with the presence or absence of the SATAC.

The segregation behavior of the SATAC was consistent with a normal chromosome; a typical segregation pattern was observed, indicating the functional equivalency of the SATAC to resident chromosomes in terms of genetic behavior. Thus, this result shows that seedlings displayed homozygous, hemizygous or null phenotypes at a predicted Mendelian frequency. The results also demonstrate the SATAC was maintained not only during mitosis, but meiosis as well. Similar observations were found with tobacco plants containing a SATAC, and sexual transmission of the SATAC via sexual crossing.

FIGURE 1 – Fluorescence in situ hybridization analysis (FISH) of Canola (*Brassica Napus*) root tips from a SATAC Line showing segregation of the SATAC



III. Conclusion

The results of the experiments provided herein demonstrate that by following the teachings of the specification and employing standard methods, a plant satellite artificial chromosome can be generated and selected within a cell. The resulting plant satellite artificial chromosome is stably maintained and can be transmitted during growth and regeneration of plants. Accordingly, the description of a plant satellite artificial chromosome provided in the above-captioned application is sufficiently detailed such that I and other scientists involved in the work were readily able to generate and identify a plant satellite artificial chromosome and distinguish it in a background of plant chromosomes based on the description in the application. The above studies also demonstrate the stability of the SATAC and its transmission in mitosis and meiosis is normal, and that the SATAC is generationally stable. Thus, the SATAC has a functional centromere. Thus, by following the teachings in the above-captioned application one can readily generate a plant satellite artificial chromosome.

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I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Feb 05 2008
Date

Steven F. Fabijanski